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Abstract

Dysfunctional variants of the innate immune cell surface receptor TREM2 (triggering receptor expressed on myeloid cells-2) were identified as major genetic risk factors for Alzheimer's disease and other neurodegenerative conditions. Here we assessed a possible involvement of TREM2 in prion disease. We report that TREM2 expression by microglia is significantly upregulated upon prion infection. However, depletion of TREM2 did not affect disease incubation time and survival after intracerebral prion infection. Interestingly, markers of microglial activation were attenuated in prion-infected TREM2$^{-/-}$ mice, suggesting an involvement of TREM2 in prion-induced microglial activation. Further phenotype profiling of microglia revealed that TREM2 deficiency did not change microglial phenotypes. We conclude that TREM2 is involved in prion-induced microglial activation, but does not noticeably modulate the pathogenesis of experimental prion infections.

Key words

TREM2, prion disease, neuroinflammation, microglial activation, pathogenesis

1 Introduction

Prion diseases are fatal neurodegenerative disorders, which include scrapie in sheep and goats, bovine spongiform encephalopathy in cows, as well as Creutzfeldt-Jakob disease (CJD) and kuru in humans (Aguzzi, et al., 2013). Prion diseases are, to date, incurable. The histopathological features of prion diseases encompass neuronal loss, gliosis, spongiform changes and the deposition of PrP^{Sc}, an abnormal isoform of the cellular prion protein (PrP^{C}) encoded by the *Prnp* gene. A crucial feature that distinguishes prion diseases from all other neurodegenerative diseases is transmissibility between individuals. The infectious agent is composed of PrP^{Sc}, which can act as template to induce the conversion of PrP^{C} into infectious stereoisoforms (Prusiner, 1982). Therefore, elimination of PrP^{C} abolishes the conversion of PrP^{C} into PrP^{Sc}, and $Prnp^{-/-}$ mice are resistant to prion infections.

Pulse-chase studies of prion infectivity in *Prnp*-ablated mice, which lack all PrP^{C}, have shown that prions can be cleared *in vivo* with surprising efficiency (Sailer, et al., 1994). It was then found that microglia, the most prevalent immune cells of the brain, act as the main scavenger of prions. In prion-infected cerebellar organotypic cultured slices, depletion of microglia vastly enhances prion deposition and leads to increased prion infectivity (Falsig, et al., 2008). The opsonin Mfge8 is instrumental for microglia-dependent clearance of prions, possibly by bridging phospholipids trapped within PrP^{Sc} to integrins expressed by microglia (Kranich, et al., 2010). However, the impact of Mfge8 is highly variable among different mouse strains, suggesting that additional molecules (which may be polymorphic in the mouse) play roles similar to Mfge8 in prion clearance (Aguzzi and Zhu, 2012). The identification of such additional molecules is of practical importance, since they may represent therapeutic targets for this group of diseases.

Triggering receptor expressed on myeloid cells 2 (TREM2) is an innate immune cell surface receptor that facilitates phagocytosis of apoptotic neurons and bacteria, thereby quenching inflammation (N'Diaye, et al., 2009,Takahashi, et al., 2005). Loss-of-function mutations in TREM2 occur in patients suffering from Nasu-Hakola disease, which is characterized by

bone cysts and early-onset progressive dementia (Paloneva, et al., 2002). These findings have prompted studies of TREM2 in other neurodegenerative conditions such as Alzheimer's disease (AD). TREM2 has been reported to be upregulated in mouse models of AD (Frank, et al., 2008), and – crucially – two independent studies have found that variants of TREM2 are associated with a higher risk of developing AD (R. Guerreiro, et al., 2013b,Jonsson, et al., 2013). These findings have spurred numerous studies addressing the association between TREM2 and AD and other neurodegenerative diseases. Although negatives have been reported (Jiao, et al., 2014,Ma, et al., 2014,Miyashita, et al., 2014,Ruiz, et al., 2014,Yu, et al., 2014), the association between TREM2 variants and AD has been confirmed by most studies (Benitez, et al., 2013,Cuyvers, et al., 2014,Giraldo, et al., 2013,Gonzalez Murcia, et al., 2013,Luis, et al., 2014,Slattery, et al., 2014). Moreover, TREM2 variants were also found to be associated with frontotemporal dementia-like syndrome or FTD (Borroni, et al., 2014,R. Guerreiro, et al., 2013a,R.J. Guerreiro, et al., 2013,Le Ber, et al., 2014,Rayaprolu, et al., 2013,Thelen, et al., 2014), Parkinson's disease (PD) (Rayaprolu, et al., 2013), and sporadic amyotrophic lateral sclerosis (ALS) (Cady, et al., 2014). These findings raise the question whether the dysregulation of crucial immunological functions associated with TREM2 variants may be a general causative factor in a broad spectrum of neurodegenerative diseases.

TREM2 has been reported to function as a phagocytic receptor for neuronal debris and bacteria (N'Diaye, et al., 2009,Takahashi, et al., 2005). Therefore, it is conceivable that TREM2 may also facilitate the phagocytosis and clearance of extracellularly deposited protein aggregates, such as Aβ plaques. Therefore, loss-of-function mutations in TREM2 may lead to unimpeded protein aggregate deposition and result in progression of the respective diseases. Indeed, missense TREM2 mutations associated with FTD and FTD-like syndrome were found to reduce TREM2 protein maturation, thereby abolishing its shedding by ADAM proteases and impairing the phagocytic activity of TREM2-expressing cells (Kleinberger, et al., 2014). However, others reported that the depletion of one allele of

TREM2 in the APPPS1 mouse model did not affect Aβ deposition, even if TREM2 heterozygosity altered the microglial response in these mice (Ulrich, et al., 2014). Therefore, it remains unknown if, and to what extent, complete depletion of TREM2 affects Aβ catabolism. Interestingly, overexpression of TREM2 in a mouse model of AD afforded protection against AD progression through the modulation of microglia functions (Jiang, et al., 2014a).

Here we searched for a possible role of TREM2 in a mouse model of prion disease. We found that TREM2 expression in microglia was significantly upregulated by prion infection, and microglial activation was significantly attenuated in prion-infected TREM2$^{-/-}$ mice. However, ablation of TREM2 did not change the incubation time and the salient biochemical and pathophysiological features after intracerebral inoculation of prions. Our study suggests that TREM2 is involved in prion-induced microglial activation, but does not contribute to prion pathogenesis in the mouse brain.

2 Material and methods

2.1 Ethical statement

All animal experiments were carried out in strict accordance with the Rules and Regulations for the Protection of Animal Rights (Tierschutzgesetz and Tierschutzverordnung) of the Swiss Bundesamt für Veterinärwesen and were preemptively approved by the Animal Welfare Committee of the Canton of Zürich (permit # 41/2012).

2.2 Animals

TREM2$^{-/-}$ mice carrying a targeted deletion of exon 3 and 4, which encodes portions of transmembrane and cytoplasmic domains of TREM2 (Turnbull, et al., 2006) were first backcrossed to C57BL/6J to obtain TREM2$^{+/-}$ offspring. TREM2$^{+/-}$ mice were then intercrossed to obtain TREM2$^{-/-}$, TREM2$^{+/-}$ and TREM2$^{+/+}$ (WT) littermates, which were used for the experiments described here. For microglia depletion experiments, we used Tg20/CD11b-HSVTK mice (Heppner, et al., 2005).

2.3 Prion inoculation

Mice were intracerebrally (i.c) inoculated with 30 µl of brain homogenate diluted in PBS with 5% BSA and containing 3 x 5 log LD50 units of the Rocky Mountain Laboratories scrapie strain (passage 6, thus called RML6). Scrapie was diagnosed according to clinical criteria (ataxia, kyphosis, priapism, and hind leg paresis). Mice were sacrificed on the day of onset of terminal clinical signs of scrapie.

2.4 Organotypic slice cultures and microglia depletion.

Cerebellar organotypic slices were prepared from 9-11-day old pups and maintained according to previously published protocols (Falsig, et al., 2008). In Tg20/CD11b-HSVTK brain slices, microglia were depleted by adding ganciclovir from 0 to 21 days *in vitro*. Slices were then infected with 10^{-4} dilution of RML6 prions or non-infectious brain homogenates

from CD1 mice (NBH). Five weeks postinoculation, slices were harvested and total RNA was extracted.

2.5 Quantitative real-time PCR (qRT-PCR)

Total RNA from brain or cultured slices was extracted using TRIzol (Invitrogen) according to the manufacturer's instruction. The quality of RNA was analyzed by Bioanalyzer 2100 (Agilent Technologies), RNAs with RIN>6 were used for cDNA synthesis. cDNA were synthesized from ~1 µg total RNA using QuantiTect Reverse Transcription kit (QIAGEN) according to the manufacturer's instruction. Quantitative real-time PCR (qRT-PCR) was performed using the SYBR Green PCR Master Mix (Roche) on a ViiA7 Real-Time PCR system (Applied Biosystems). Expression levels were normalized using GAPDH. The sequence of qRT-PCR primers are in table 1.

2.6 Western blot analysis

To detect PrP^C in the $TREM2^{-/-}$, $TREM2^{+/-}$ and $TREM2^{+/+}$ (WT) brains, hemisphere of brains were homogenized with buffer PBS containing 0.5% Nonidet P-40 and 0.5%CHAPSO. Total protein concentration was determined using the bicinchoninic acid assay (Pierce). ~8 µg proteins were loaded and separated on a 12% Bis-Tris polyacrylamide gel (NuPAGE, Invitrogen) and then blotted onto a nitrocellulose membrane. Membranes were blocked with 5% wt/vol Topblock (Fluka) in PBS supplemented with 0.05% Tween 20 (vol/vol) and incubated with primary antibodies POM1 in 1% Topblock (200 ng ml^{-1}) overnight. After washing, the membranes were then incubated with secondary antibody horseradish peroxidase (HRP)-conjugated rabbit anti–mouse IgG1 (1:10,000, Zymed). Blots were developed using Luminata Crescendo Western HRP substrate (Millipore) and visualized using the Stella system (model 3000, Bio-Rad). To avoid the variation in loading, the same blots were striped and incubated with an anti-actin antibody (1:10,000, Millipore). The PrP^C signals were normalized to actin as a loading control.

To detect PrP^{Sc}, prion infected forebrains were homogenized in sterile 0.32 M sucrose in PBS. Total protein concentration was determined using the bicinchoninic acid assay (Pierce). Samples were adjusted to 20 µg protein in 20 µl and digested with 25 µg ml^{-1} proteinase K in digestion buffer (PBS containing 0.5% wt/vol sodium deoxycholate and 0.5% vol/vol Nonidet P-40) for 30 min at 37°C. PK digestion was stopped by adding loading buffer (Invitrogen) and boiling samples at 95°C for 5 min. Proteins were then separated on a 12% Bis-Tris polyacrylamide gel (NuPAGE, Invitrogen) and blotted onto a nitrocellulose membrane. POM1 and horseradish peroxidase (HRP)-conjugated rabbit anti–mouse IgG1 were used as primary and secondary antibodies, respectively. Blots were developed using Luminata Crescendo Western HRP substrate (Millipore) and visualized using the FUJIFILM LAS-3000 system. To detect IBA-1 and GFAP in prion-infected brains by western blotting, 20 µg of total brain protein were loaded and anti-IBA-1 antibody (1:1000; Wako Chemicals GmbH, Germany) and anti-GFAP antibody (1:10,000; Abcam) were used. Actin was used as loading control.

2.7 Immunohistochemistry

Formalin-fixed tissues were treated with concentrated formic acid for 60 min to inactivate prion infectivity and embedded in paraffin. Paraffin sections (2 µm) of brains were stained with hematoxylin/eosin (HE). After deparaffinization through graded alcohols, antibodies GFAP (1:300; DAKO, Carpinteria, CA) for astrocytes were applied and visualized using standard methods, IBA-1 (1:1000; Wako Chemicals GmbH, Germany) was used for highlighting activated microglial cells. Stainings were visualized using DAB (Sigma-Aldrich), hematoxylin counterstain was subsequently applied. For the histological detection of partially proteinase K-resistant prion protein deposition, deparaffinized sections were pretreated with formaldehyde for 30min and with 98% formic acid for 6 min, and then washed in distilled water for 30 min. Sections were incubated in Ventana buffer and stains were performed on a NEXEX immunohistochemistry robot (Ventana instruments, Switzerland) using an IVIEW DAB Detection Kit (Ventana). After incubation with protease 1 (Ventana) for 16 min, sections

were incubated with anti-PrP SAF-84 (SPI bio, A03208, 1:200) for 32 min. Sections were counterstained with hematoxylin. Sections were imaged using a Zeiss Axiophot light microscope. Quantification of IBA-1 and GFAP staining was performed on acquired images; regions of interest were drawn and the percentage of brown IBA-1 and GFAP staining over the total area was quantified using scripts developed in-house. The operator was blind to the genotype and treatment of the analyzed tissues.

2.8 Statistical analysis

Results are presented as the mean of replicas ± standard deviation (SD). Statistical significance between experimental groups was assessed using an unpaired Student's t-Test. Incubation times were analyzed using the Kaplan-Meier method and compared to gender-matched TREM2$^{+/+}$ (WT) littermates using the logrank test. *p*-values <0.05 were considered statistically significant.

3 Results

3.1 Prion infection upregulates TREM2 and DAP12 expression by microglia in mouse brain

TREM2 has been described to be upregulated in AD mouse models (Frank, et al., 2008). To determine whether TREM2 expression is altered by prion infection, we carried out quantitative reverse-transcription PCR (qRT-PCR) on mRNA isolated from terminally sick wild-type (WT) C57BL/6 mice infected with the Rocky Mountain Laboratory strain of mouse-adapted scrapie prions (passage #6, inoculum hence nicknamed RML6), age-matched WT C57BL/6 mice inoculated with non-infectious brain homogenates (NBH), and *Prnp*$^{-/-}$ mice infected with RML6. We found TREM2 mRNA to be significantly upregulated (6.5-fold) in RML6-infected C57BL/6 mice compared to NBH-inoculated controls (Figure 1A). Prion-resistant *Prnp*$^{-/-}$ mice (Bueler, et al., 1993) inoculated with RML6 did not show any upregulation of TREM2 expression (Figure 1A), which is in accordance with the absence of prion pathology in those mice. Therefore, similarly to what was reported for AD mouse models, TREM2 is upregulated in a mouse model of prion diseases. Interestingly, the adapter protein DAP12 that is associated with TREM2 was also significantly upregulated (6.1-fold) in prion infected brains (Figure 1A).

Myeloid cells are the main sources of TREM2, and microglia are thought to be the main producers of TREM2 within the brain (Jiang, et al., 2014b). To assess the contribution of microglia to the expression of TREM2 in the brain after prion infection, we prepared cerebellar organotypic cultured slices (COCS) from transgenic mice that express the herpes simplex virus thymidine kinase under the promoter of CD11b and overexpress PrP^{C} (Tg20/CD11b-HSVTK). Treatment of such COCS with ganciclovir (GCV) essentially removes all microglia (Heppner, et al., 2005).

After prion infection, microglia-containing slices expressed significantly more TREM2 (2.5-fold) than NBH-exposed slices, similarly to what we had observed *in vivo*. However, after microglia depletion, TREM2 was barely detectable in both RML and NBH treated slices (Figure 1B). Similarly, DAP12 was also significantly upregulated (2.3-fold) in prion infected

microglia-containing slices and was barely detectable when microglia were depleted (Figure 1B). The small amounts of TREM2 and DAP12 remaining in microglia-depleted samples (<0.1% of NBH) originated from residual microglia, since several other microglial markers (CD11b, CSF1R, CX3CR1) showed similar expression pattern as TREM2 and DAP12 in microglia-depleted slices (Figure 1C). Collectively, these results indicate that microglia are not only the main cell type expressing TREM2 and DAP12 in the brain under physiological conditions, but they are also the dominant cell type that is induced to express TREM2 and DAP12 after prion infection.

3.2 TREM2 deficiency does not affect PrP^C expression

The expression level of PrP^C is the primary factor influencing prion incubation time. Reduction of PrP^C by 50% (in *Prnp*$^{+/-}$ hemizygous mice) drastically prolongs the incubation time of prion disease (Bueler, et al., 1993), whereas overexpression of PrP^C markedly shortens prion disease progression (Fischer, et al., 1996). To evaluate whether TREM2 deficiency could affect PrP^C expression, we first measured *Prnp* mRNA levels in the brains of TREM2$^{-/-}$, TREM2$^{+/-}$ and TREM2$^{+/+}$ (WT) littermates by qRT-PCR. We did not observe any significant differences in *Prnp* mRNA between these three groups (Figure 2A). As expected, TREM2 mRNA was about 50% in TREM2$^{+/-}$ mice compared to TREM2$^{+/+}$ mice, and undetectable in TREM2$^{-/-}$ mice (Figure 2A). We also assessed the PrP^C protein content of brains from TREM2$^{-/-}$, TREM2$^{+/-}$ and TREM2$^{+/+}$ (WT) littermates by western blot, and found that all three groups of mice expressed similar levels of PrP^C (Figure 2B). Hence TREM2 deficiency does not affect PrP^C expression in mouse brain.

3.3 Similar prion pathogenesis in TREM2$^{-/-}$ and wild-type littermates after intracerebral inoculation

To determine if TREM2 is involved in prion pathogenesis in the brain, we inoculated RML6 intracerebrally into TREM2$^{-/-}$, TREM2$^{+/-}$ and TREM2$^{+/+}$ (WT) littermates. The incubation time

was determined by measuring the time lapse between inoculation and the terminal stage of prion disease. Since gender was reported to be a factor affecting prion incubation time (Akhtar, et al., 2011), we compared female and males with different genotypes separately. However, we observed similar incubation times in all three groups (Figure 3A) in both females and males.

TREM2 is considered as an important receptor for phagocytosis, which is the main mechanism of prion elimination from the brain. In order to assess whether TREM2 is involved in prion clearance, we analyzed PrP^{Sc} deposition in terminally scrapie-sick mice. We did not observe any statistically significant differences in the total amount of proteinase K (PK) resistant prion protein (PrP^{Sc}) between $TREM2^{+/+}$, $TREM2^{+/-}$, and $TREM2^{-/-}$ brains (Figure 3B). Prion infectivity of terminally sick mouse brains was determined using a cell-based end-point dilution method, and showed similar prion titers in all three genotypes (Figure 3C). Histological examination of the brains at the terminal stage of disease revealed similar lesion patterns, with equivalent PrP^{Sc} deposit levels (Figure 3B and 3D) and astrogliosis in the three groups (Figure 3D, 3E and 3F). Therefore, although prion infection induced upregulation of TREM2 expression by microglia, TREM2 did not exert a discernible impact onto various parameters of prion disease pathogenesis.

3.4 $TREM2^{-/-}$ mice displayed attenuated microglial activation upon prion infection

TREM2 has been found to facilitate the phagocytosis of apoptotic neurons and to suppress inflammatory responses (Takahashi, et al., 2005). To determine whether depletion of TREM2 affects the basal inflammatory levels in the brain, we first measured the proinflammatory cytokines TNFα, IL-1β and IL-6 in the brains of $TREM2^{-/-}$, $TREM2^{+/-}$ and $TREM2^{+/+}$ (WT) littermates by qRT-PCR. Interestingly, we did not detect significant differences between the groups (Figure 4A). Therefore, we conclude that TREM2 deficiency has a limited impact, if any, on the baseline inflammatory status of mouse brains. We also analyzed the expression of proinflammatory cytokines TNFα, IL-1β and IL-6 in the brains of prion-infected $TREM2^{-/-}$,

TREM2$^{+/-}$ and TREM2$^{+/+}$ (WT) littermates. Consistently with previous studies, these cytokines were all upregulated upon prion infection (Figure 4A). However, ablation of TREM2 did not significantly alter the expression of these cytokines (Figure 4A).

Neuroinflammation including microglial activation is one of the hallmarks of prion pathogenesis, and it has been suggested that activated microglia may damage brain structures – thereby contributing to neurodegeneration. As suggested by previous publications and confirmed by our own experiments, TREM2 is mainly expressed by microglia in the brain. To test whether TREM2 deficiency affects prion-induced microglial activation, we assessed microglial activation in the brains of terminally sick mice by IBA-1 immunohistochemical staining. Interestingly, we found significantly reduced microglial density in TREM2$^{-/-}$ mice compared to their TREM2$^{+/+}$ (WT) littermates (Figure 4B), suggesting that proliferation of microglia in TREM2$^{-/-}$ mice was diminished. IBA-1 western blot of brain tissue from terminally sick mice confirmed that TREM2$^{-/-}$ mice showed less microglial activation (Figure 4C). We conclude that TREM2 does play a role in prion-dependent microglial activation, even if it does not control prion pathogenesis.

Analogous to extracerebral macrophages, microglia can be polarized into two different phenotypes termed M1 and M2. M1-polarized microglia show pro-inflammatory features and express TNFα, IL-1β, IL-6, inducible nitric oxide synthase (iNOS), and arginase 2 (Arg2), whereas M2 microglia display a phagocytic behavior and express FIZZ1, Ym1/2 and arginase 1 (Arg1). To better characterize the role of TREM2 in prion induced microglial activation, we carried out qRT-PCR for genes reflecting the phenotype of microglia in TREM2$^{-/-}$, TREM2$^{+/-}$ and TREM2$^{+/+}$ (WT) mice infected with RML6 prions or exposed to non-infectious brain homogenates, respectively. We did not observe any statistically significant differences between the three groups (Figure 4D). Therefore, TREM2 deficiency did not alter the baseline microglial polarization and did not change the prion-induced microglia neuroinflammatory phenotype.

4 Discussion

Neuroinflammation has been discussed as a pathogenetic factor in neurodegeneration for many years, but it has been difficult to corroborate this concept. The finding that TREM2 variants act as strong genetic risk factors for Alzheimer's disease and many other neurodegenerative diseases (Benitez, et al., 2013,Borroni, et al., 2014,Cady, et al., 2014,Cuyvers, et al., 2014,Giraldo, et al., 2013,Gonzalez Murcia, et al., 2013,R. Guerreiro, et al., 2013a,R. Guerreiro, et al., 2013b,R.J. Guerreiro, et al., 2013,Jonsson, et al., 2013,Le Ber, et al., 2014,Rayaprolu, et al., 2013) represents the most direct evidence to date that immune functions can modulate the course of these diseases. According to the currently prevailing view, loss-of-function mutations in TREM2 may result in unconstrained protein aggregate deposition and accelerate progression of these diseases.

Because prion infections go along with conspicuous extracellular aggregates of PrP^{Sc}, it seemed worthwhile to test whether experimental prion diseases is affected by the TREM2 state of the host. In contrast to most other models of neurodegeneration, prion-infected mice undergo a highly stereotypic course of disease with a well-defined incubation and survival time. These properties greatly facilitate the objective and precise assessments of the impact of genetic manipulations on prion pathogenesis. We opted to investigate the model of mouse-adapted scrapie, a prion disease which not only sports highly predictable and homogeneous incubation times but also robust endpoints and reliable biomarkers.

Prion infections are among the most powerful stimuli for microglia proliferation, and indeed we found that TREM2 is conspicuously upregulated (6.5-fold) upon prion infection. This finding is consistent with reports that within the CNS, TREM2 is primarily expressed by microglia, but it may reflect correlation rather than causality. We therefore investigated this issue further by using the CD11b-HSVTK transgenic mice, which express the tyrosine kinase of Herpes simplex virus under the transcriptional control of the myeloid CD11b promoter. Exposure of CD11b-HSVTK organotypic cerebellar slices to ganciclovir results in its phosphorylation to a nucleoside analogue that poisons cells undergoing DNA synthesis

(including mitochondrial DNA). This results in rapid, selective and complete ablation of all microglia. These experiments provided more definitive proof that microglia are not only the main source of TREM2 expression in mouse brain, but also the dominant cell type that is stimulated to express TREM2 after prion infection. We noticed that the extent of TREM2 and DAP12 upregulation in Tg20/CD11b-HSVTK COCS was less abundant than in the *in vivo* brain samples. We ascribe this phenomenon to the process of COCS generation, which results in sizeable microglial activation by itself. Such pre-activated microglia may be unable to demonstrate a higher degree of activation upon additional stimuli.

Although TREM2 expression was conspicuously upregulated by prion infection, its ablation did not alter the speed of prion disease progression. Moreover, PrP^{Sc} deposition and astrogliosis were similar in all three groups. Since the phagocytic activity of microglia is a well-established limiting factor for prion propagation within the brain(Falsig, et al., 2008,Kranich, et al., 2010,Nuvolone, et al., 2013), the unchanged PrP^{Sc} deposition and prion infectivity titer suggested that the phagocytosis of prions by $TREM2^{-/-}$ microglia was not markedly affected. However, TREM2 seems to be involved in microglial activation, as the prion-induced enhancement of IBA-1 reactivity was attenuated in $TREM2^{-/-}$ mice. This is consistent with a previous report that APPPS1 mice heterozygous for TREM2 exhibited decreased number and size of plaque-associated microglia (Ulrich, et al., 2014). The latter data confirm that microglial activation was attenuated upon TREM2 hemizygosity, yet their phagocytic capacity was not obviously compromised. In a study on stroke model, microglial activity in the subacute phase was also reduced in $TREM2^{-/-}$ mice although the lesion size was not affected (Sieber, et al., 2013). Collectively, these results suggest that TREM2 is an important mediator for microglial activation. The absence of effect of TREM2 on Aβ plaques and prion load is surprising; it suggests that the microglial activation state may not necessarily correlate with their phagocytic capacity. Together with our previous findings that depletion of microglia enhances prion replication in organotypic cerebellar slices (Falsig, et al., 2008), we hypothesize that the availability of microglia, and perhaps a basal level of

activation, may be crucial for phagocytosis, whereas further activation does not appear to augment the clearance of prions. Accordingly, lipopolysaccharide-stimulated microglial activation did not enhance further phagocytosis of prions (Hughes, et al., 2010).

Further microglia profiling indicated that TREM2 did not conspicuously affect microglial polarization under physiological conditions and after prion infection. We did identify minor trends in some markers which may have arguably reached statistical significance if a larger collective of animals had been studied. Notwithstanding this caveat, our results suggest that the action of TREM2 on microglia is subtle and does not exert any immediately obvious impact onto its functionality. In particular, its effect onto the pathogenesis and course of prion infection is negligible.

One possible limitation to our study is that we did not exclude the effect of TREM2 deficiency on peripheral macrophages that might invade the central nervous system (CNS) under pathological conditions. Although there is no evidence that TREM2 affects the recruitment of macrophages from the periphery to the CNS, this question remains open and may merit further investigations.

Along with the failure to find any association between specific TREM2 allelotypes and sporadic CJD (Slattery, et al., 2014), the findings described here add to the evidence that TREM2 is not a major determinant of prion pathogenesis. This fact is unexpected and sets prions apart from other neurodegenerative diseases such AD, FTD, PD, and ALS.

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Figure captions

Figure 1: Upregulation of TREM2 and DAP12 after prion infection. (A) qRT-PCR for TREM2 and DAP12 expression. Age-matched wild-type C57BL/6 mice were intracerebrally inoculated with prion-containing or non-infectious CD1 mouse brain homogenates (RML and NBH, respectively; days after inoculation: ~170). Prion-inoculated *Prnp*$^{-/-}$ mice served as additional controls. *TREM2* and *DAP12* mRNAs were significantly upregulated in prion-infected WT mice, but not in prion resistant *Prnp*$^{-/-}$ mice (n=4, ***: p<0.001, ****: p<0.0001). (B) Cerebellar organotypic cultured slices from Tg20/CD11b-HSVTK mice were treated with ganciclovir (GCV) to deplete microglia. GCV-treated and untreated controls were infected with RML6 prions or NBH. In GCV-treated slices, TREM2 and DAP12 were barely detectable, indicating that TREM2 and DAP12 are mainly contributed by microglia. Without GCV, TREM2 and DAP12 expression was again upregulated upon prion infection (n=4, ** p<0.01, ***: p<0.001, **** p<0.0001). (C) qRT-PCR for CD11b, CSF1R and CX3CR1 expression on Tg20/CD11b-HSVTK cerebellar organotypic cultured slices. Similarly to TREM2 and DAP12, these microglial markers were barely detectable in microglia-depleted slices, yet were upregulated in prion infected, microglia-containing slices (n=4, *: p<0.05, ** p<0.01, ***: p<0.001, **** p<0.0001). Data are presented as the mean ± SD.

Figure 2: Unaltered PrP^{C} expression in TREM2 deficient mice. (A) *Prnp* mRNA levels in TREM2$^{+/+}$ (WT), TREM2$^{+/-}$ and TREM2$^{-/-}$ littermates were similar (n=3, n.s p>0.05). *TREM2* mRNA level was about half in TREM2$^{+/-}$ littermates (n=3, ** p<0.01) and undetectable in TREM2$^{-/-}$ littermates. (B) Western blot showing similar PrP^{C} concentrations in brains of TREM2$^{+/+}$ (WT), TREM2$^{+/-}$, and TREM2$^{-/-}$ littermates (n=4, n.s p>0.05 Data are presented as mean ± SD.

Figure 3: Unaltered pathogenesis in TREM2-ablated mice after intracerebral prion infection. (**A**) Similar survival curves of gender-matched $TREM2^{+/+}$, $TREM2^{+/-}$ and $TREM2^{-/-}$ littermates intracerebrally inoculated with RML6 prions (n=3-7, n.s: $p>0.05$). (**B**) Left: Western blot for proteinase K resistant PrP^{Sc} in terminally sick mouse brains. Right: densitometric quantification failed to reveal differences between $TREM2^{+/+}$ (WT), $TREM2^{+/-}$ and $TREM2^{-/-}$ littermates (n=3, n.s: $p>0.05$). (**C**) Similar prion titers in terminally sick mouse brains from $TREM2^{+/+}$ (WT), $TREM2^{+/-}$, and $TREM2^{-/-}$ littermates (n=3, n.s: $p>0.05$). (**D**) Representative histology of terminally sick mouse brains from $TREM2^{+/+}$ (WT), $TREM2^{+/-}$ and $TREM2^{-/-}$ littermates. There was no obvious difference between the three groups in lesion patterns, vacuolation, PrP^{Sc} deposition. Scale bars are 100μm. (**E**) Quantification of GFAP staining for terminally sick mouse brains from $TREM2^{+/+}$ (WT), $TREM2^{+/-}$ and $TREM2^{-/-}$ littermates. There was no obvious difference between the three groups in astrogliosis (n=5~6, n.s: $p>0.05$). (**F**) Western blot of brains from terminally sick $TREM2^{+/+}$ (WT), $TREM2^{+/-}$ and $TREM2^{-/-}$ littermates showed that GFAP expression was similar in $TREM2^{+/+}$ (WT), $TREM2^{+/-}$ and $TREM2^{-/-}$ littermates (n=3, n.s: $p>0.05$). Data are presented as the mean ± SD.

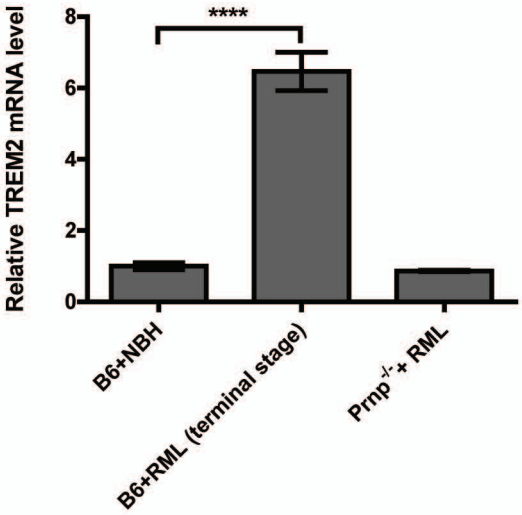
Figure 4: TREM2 deficiency attenuated microglial activation upon prion infection, but did not change the expression of inflammatory markers and the microglial phenotype. (**A**) The mRNA levels of the inflammatory cytokines, TNFα, IL-1β and IL-6, were upregulated upon prion infection, but were unaffected by TREM2 ablation (n=3, n.s: $p>0.05$). (B) Representative IBA-1 immunohistochemical staining on cortex and hippocampus of terminally sick mouse brains from $TREM2^{+/+}$ (WT), $TREM2^{+/-}$ and $TREM2^{-/-}$ littermates. $TREM2^{-/-}$ mice had significantly less microglial activation than $TREM2^{+/+}$ littermates (n=5~9, ** $p<0.01$). Scale bars are 20μm. (C) Western blot of brains from terminally sick $TREM2^{+/+}$ (WT), $TREM2^{+/-}$ and $TREM2^{-/-}$ littermates showed that IBA-1 expression was linearly dependent on TREM2 gene dosage (n=3, *: $p<0.05$). (D) TREM2 deficiency did not affect the

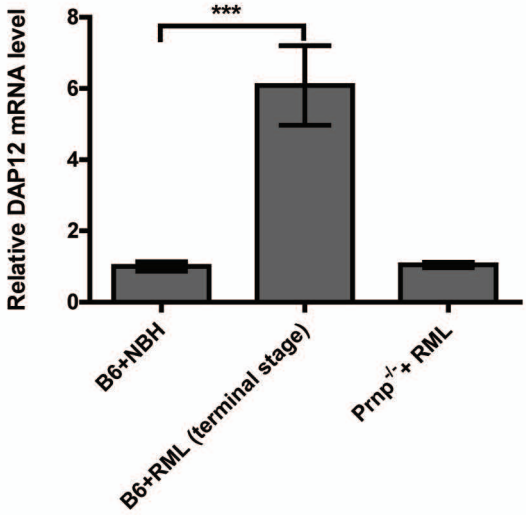
expression of pro-inflammatory M1 microglia markers (iNOS, Arg2) or anti-inflammatory M2 microglia markers (FIZZ1, Ym1/2, Arg1) under both physiological condition or upon prion infection (n=3, n.s: $p>0.05$). Data are presented as the mean ± SD.

Table 1: qRT-PCR primers

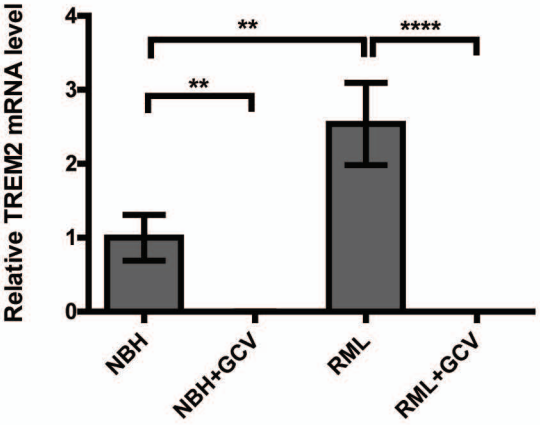
Gene	Forward primer 5'-3'	Reverse primer 5'-3'
GAPDH	CCACCCCAGCAAGGAGACT	GAAATTGTGAGGGAGATGCT
TREM2	GCACCTCCAGGAATCAAGAG	GGGTCCAGTGAGGATCTGAA
DAP12	TGCCTTCTGTTCCTTCCTGT	GGGCATAGAGTGGGCTCAT
CD11b	GTGTGACTACAGCACAAGCCG	CCCAAGGACATATTCACAGCCT
CSF1R	GCAGTACCACCATCCACTTGTA	GTGAGACACTGTCCTTCAGTGC
CX3CR1	ACCGGTACCTTGCCATCGT	ACACCGTGCTGCACTGTCC
Prnp	GCCAGTGGATCAGTACAGCA	ATCCCACGATCAGGAAGATG
*TNF*α	ACTTCGGGGTGATCGGTCCCC	TGGTTTGCTACGACGTGGGCTAC
*IL-1*β	TGCAGCTGGAGAGTGTGGATCCC	TGTGCTCTGCTTGTGAGGTGCTG
IL-6	TCCAATGCTCTCCTAACAGATAAG	CAAGATGAATTGGATGGTCTTG
FIZZ1	AGGAACTTCTTGCCAATCCA	CAGTAGCAGTCATCCCAGCA
Arg1	CAGAAGAATGGAAGAGTCAG	CAGATATGCAGGGAGTCACC
Ym1/2	CAGGGTAATGAGTGGGTTGG	CACGGCACCTCCTAAATTGT
iNOS	GTCATGGCTTCACGGGTCAG	CCAGGTCCCTGGCTAGTGCT
Arg2	TGATTGGCAAAAGGCAGAGG	CTAGGAGTAGGAAGGTGGTC

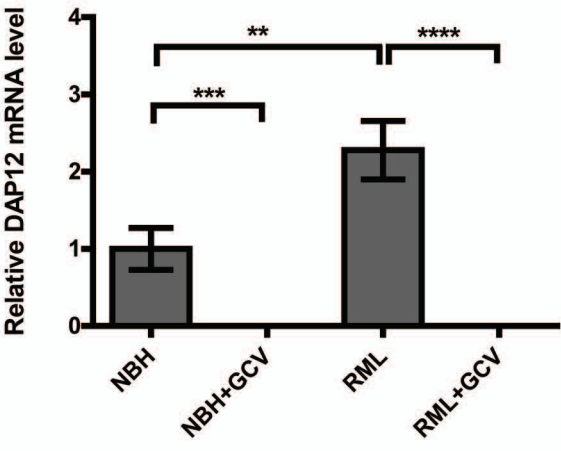
A


Relative TREM2 mRNA level
****
B6+NBH
B6+RML (terminal stage)
Prnp -/- + RML

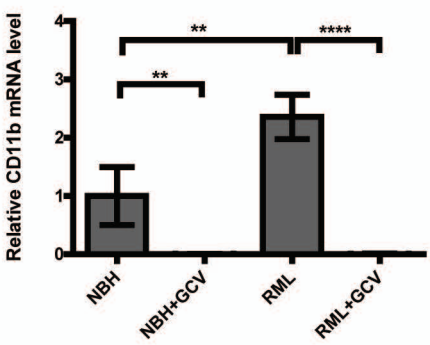

Relative DAP12 mRNA level
***
B6+NBH
B6+RML (terminal stage)
Prnp -/- + RML

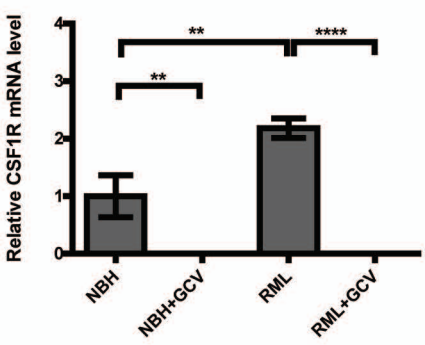
B

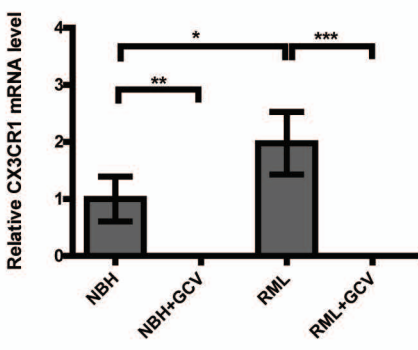

Relative TREM2 mRNA level
**
****
**
NBH
NBH+GCV
RML
RML+GCV


Relative DAP12 mRNA level
**
****
***
NBH
NBH+GCV
RML
RML+GCV

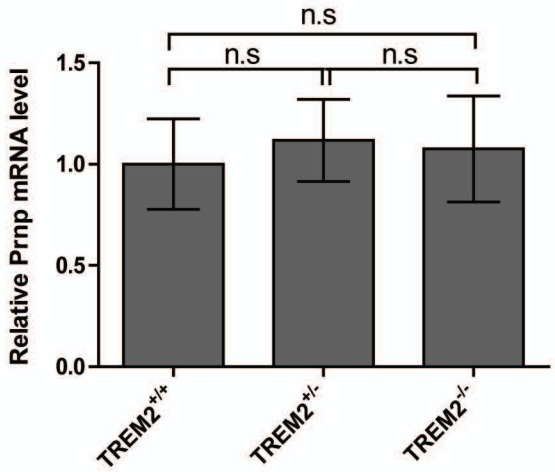
C

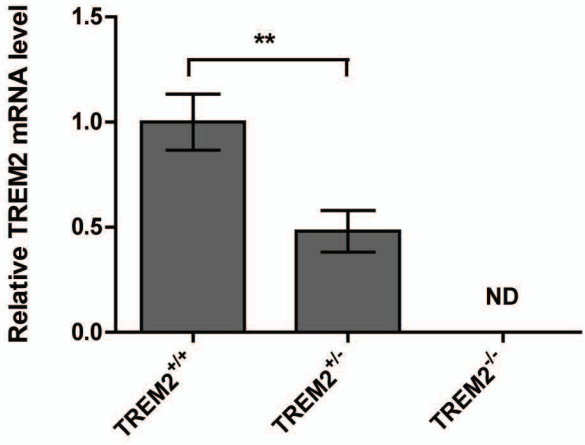

Relative CD11b mRNA level
**
****
**
NBH
NBH+GCV
RML
RML+GCV


Relative CSF1R mRNA level
**
****
**
NBH
NBH+GCV
RML
RML+GCV

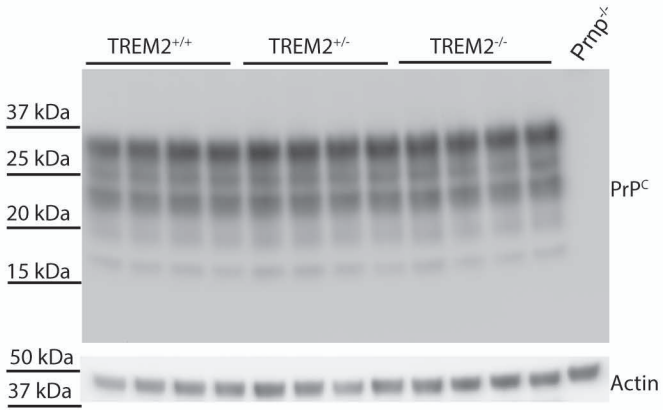

Relative CX3CR1 mRNA level
*
***
**
NBH
NBH+GCV
RML
RML+GCV

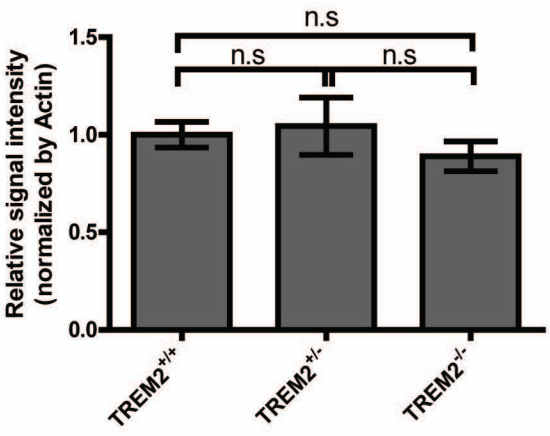
A

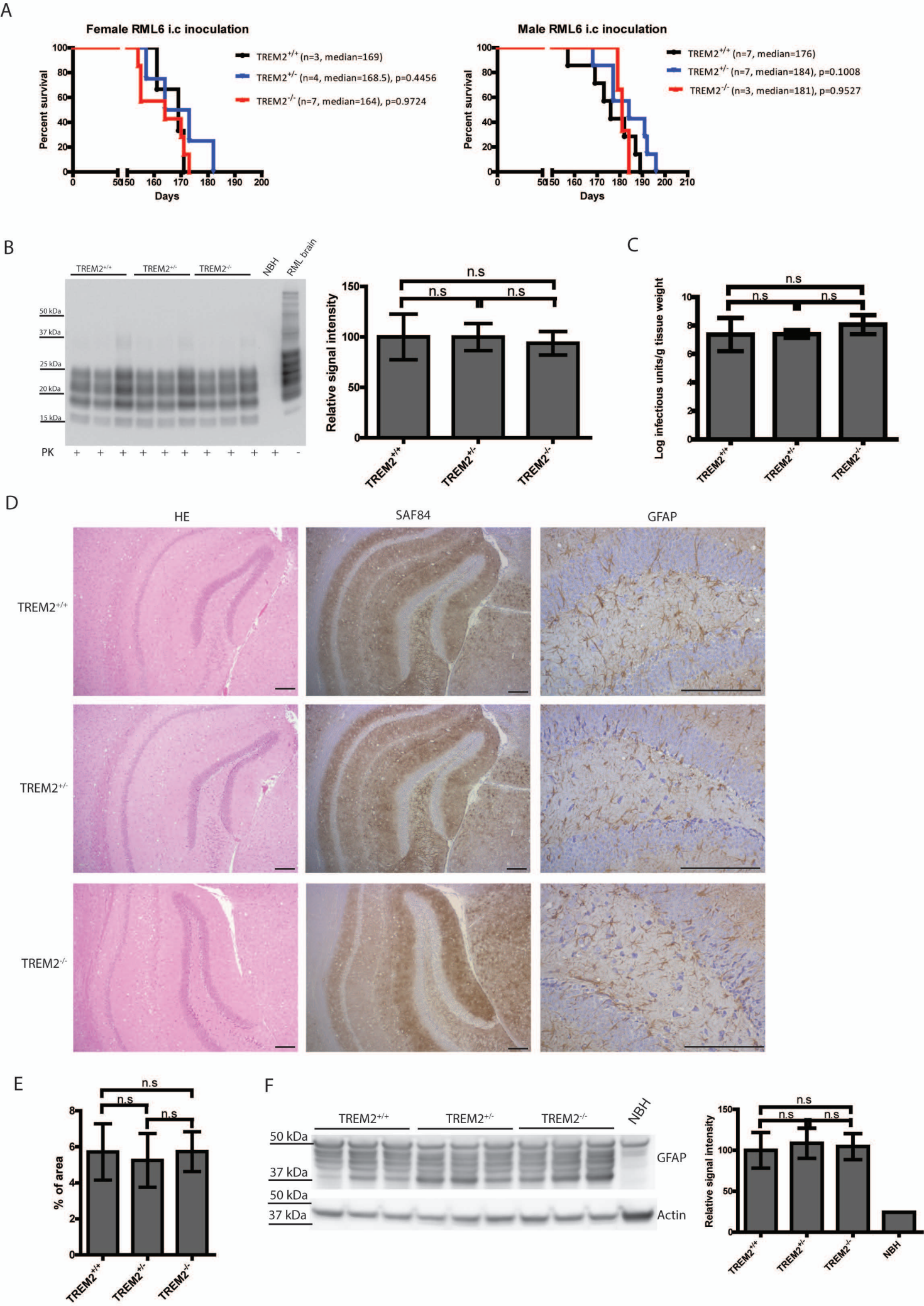

n.s
n.s
n.s
Relative Prnp mRNA level
1.5
1.0
0.5
0.0
TREM2+/+
TREM2+/-
TREM2-/-

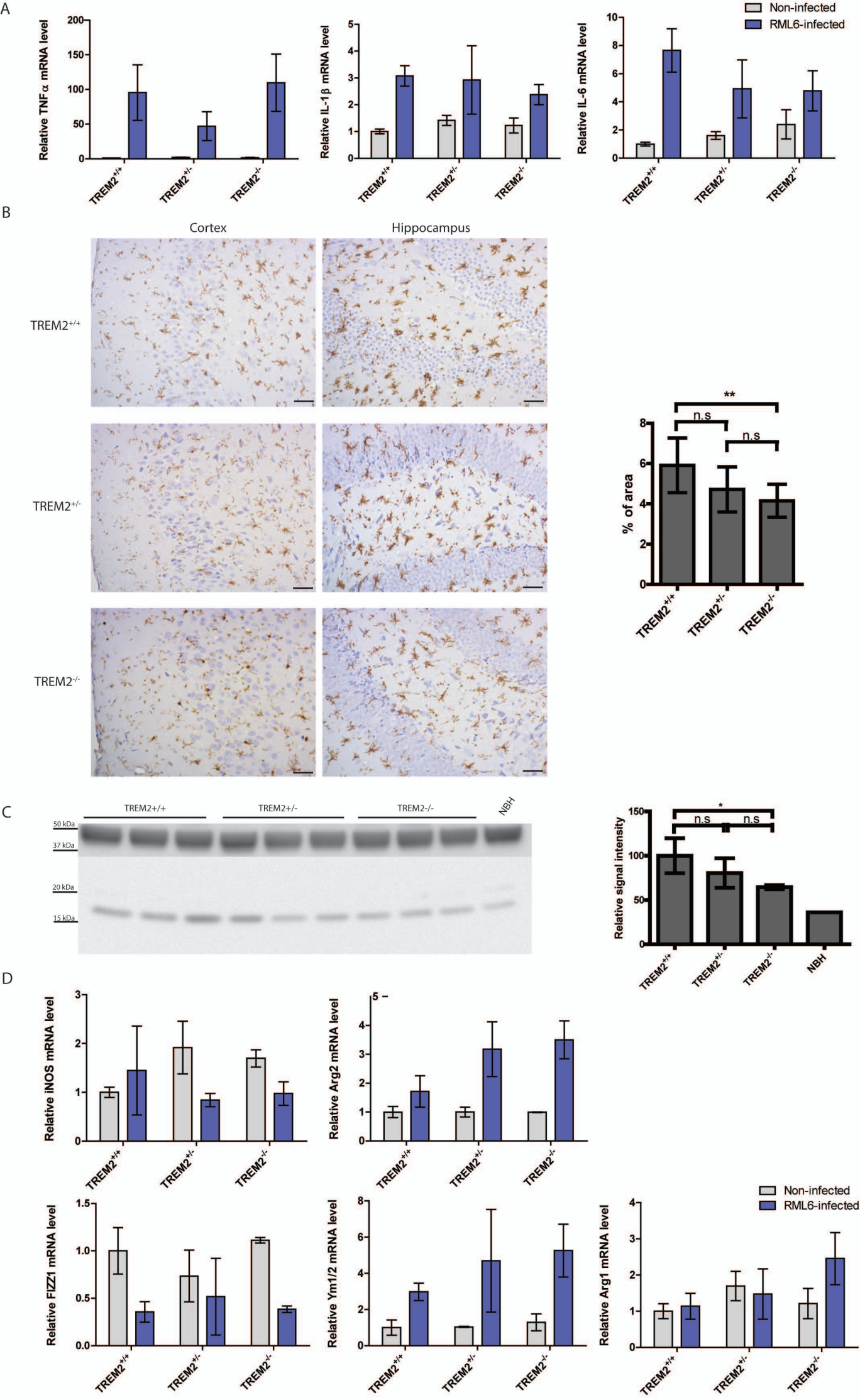

**
Relative TREM2 mRNA level
1.5
1.0
0.5
0.0
ND
TREM2+/+
TREM2+/-
TREM2-/-

B


TREM2+/+
TREM2+/-
TREM2-/-
Prnp-/-
37 kDa
25 kDa
20 kDa
15 kDa
PrPC
50 kDa
37 kDa
Actin


n.s
n.s
n.s
Relative signal intensity
(normalized by Actin)
1.5
1.0
0.5
0.0
TREM2+/+
TREM2+/-
TREM2-/-


A
Female RML6 i.c inoculation
Percent survival
Days
TREM2+/+ (n=3, median=169)
TREM2+/- (n=4, median=168.5), p=0.4456
TREM2-/- (n=7, median=164), p=0.9724
Male RML6 i.c inoculation
TREM2+/+ (n=7, median=176)
TREM2+/- (n=7, median=184), p=0.1008
TREM2-/- (n=3, median=181), p=0.9527
B
TREM2+/+
TREM2+/-
TREM2-/-
NBH
RML brain
50 kDa
37 kDa
25 kDa
20 kDa
15 kDa
PK
Relative signal intensity
n.s
C
Log infectious units/g tissue weight
D
HE
SAF84
GFAP
TREM2+/+
TREM2+/-
TREM2-/-
E
% of area
F
GFAP
Actin
50 kDa
37 kDa


A
Relative TNFα mRNA level
Relative IL-1β mRNA level
Relative IL-6 mRNA level
Non-infected
RML6-infected
TREM2+/+
TREM2+/-
TREM2-/-
B
Cortex
Hippocampus
TREM2+/+
TREM2+/-
TREM2-/-
% of area
n.s
**
C
TREM2+/+
TREM2+/-
TREM2-/-
NBH
50 kDa
37 kDa
20 kDa
15 kDa
Relative signal intensity
*
D
Relative iNOS mRNA level
Relative Arg2 mRNA level
Relative FIZZ1 mRNA level
Relative Ym1/2 mRNA level
Relative Arg1 mRNA level